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MECHANISMS OF RESISTANCE

Genetic and Functional Characterization of an MCR-3-Like Enzyme-Producing *Escherichia coli* Isolate Recovered from Swine in Brazil

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ABSTRACT A collection of 126 pigs was screened for carriage of colistin-resistant *Enterobacteriaceae* in a farm in Minas Gerais, Brazil. Out of this collection, eight colistin-resistant *Escherichia coli* isolates were recovered, including one from Minas Gerais State producing a new MCR-3 variant (MCR-3.12). Analysis of the lipopolysaccharide revealed that MCR-3.12 had a function similar to that of MCR-1 and MCR-2 as a result of the addition of a phosphoethanolamine group to the lipid A moiety. Genetic analysis showed that the *mcr-3.12* gene was carried by an IncA/C_2 plasmid and was embedded in an original genetic environment. This study reports the occurrence of the MCR-3-like determinant in South America and is the first to demonstrate the functionality of this group of enzymes as a phosphoethanolamine transferase.

KEYWORDS MCR-3, polymyxins, plasmid, swine, *mcr*

The increasing occurrence of colistin-resistant *Enterobacteriaceae* is of great concern since colistin represents one of the last-resort treatments for infections caused by carbapenem-resistant *Enterobacteriaceae* (CRE). In addition to chromosomally encoded resistance mechanisms corresponding to mutations or deletions in genes involved in the biosynthesis of the lipopolysaccharide (LPS), the acquisition of resistance through horizontal gene transfer has recently been described (1). Five different plasmid-mediated colistin resistance genes have been identified so far in *Enterobacteriaceae*, including *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5* (2–6). They code for enzymes that modify the lipid A moiety of the LPS of Gram-negative bacteria and consequently confer resistance to polymyxin B and colistin (1). To date, only MCR-1 and MCR-2 have been shown to function as phosphoethanolamine transferases (7). The *mcr-1* and *mcr-2* genes likely originate from *Moraxella* species (8), with *Moraxella pluranimalium* being the progenitor of *mcr-2* (9), *Aeromonas* spp. being the progenitor of *mcr-3*-like genes (4), and *Shewanella* spp. being the progenitor of *mcr-4*-like genes (5). The origin of the newly discovered *mcr-5* gene remains unknown (6). The high prevalence of MCR-1-producing *Escherichia coli* isolates in food-producing animals and, therefore, the high rate of occurrence of colistin-resistant isolates may be explained by the constant use of

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FIG 1 Phylogenetic tree obtained for all the identified MCR-like enzymes, including all MCR-3 variants, by the distance method using the neighbor-joining algorithm (SeaView, version 4, software). Branch lengths are drawn to scale and are proportional to the number of amino acid substitutions with 500 bootstrap replications. The distance along the vertical axis has no significance. The percent amino acid sequence identity shared between the MCR-3.12 variant and the other MCR-like enzymes is indicated in parentheses.

colistin in veterinary medicine, in particular, for the treatment of livestock (poultry, swine, and cattle) (1). To date, six *mcr-3* variants have been reported since the discovery of *mcr-3.1* in June 2017, identified from an *Escherichia coli* isolate from a healthy pig in China (4) and in a *Salmonella* isolate from human infections in Denmark (10). The *mcr-3.2* variant was identified in *E. coli* isolates from cattle in Spain (11). The *mcr-3.3* to *-3.9* variants were identified in *Aeromonas* spp. (12–15), and the *mcr-3.10* variant was identified in *E. coli* isolates from ducks in China (15). Finally, the *mcr-3.11* gene was from an *E. coli* isolate recovered from a chicken in China (unpublished; GenBank accession number MG489958.1). Even if *Aeromonas* spp. were described to be the progenitor of the *mcr-3* gene, this gene might also be found as an acquired determinant in that species (13).

Here we report on a novel *mcr-3* variant detected in an *E. coli* isolate recovered from a pig with postweaning diarrhea that had previously been treated with colistin in Brazil.

RESULTS

Characterization of a new *mcr-3* variant and susceptibility testing. Out of the 126 pig samples, 8 samples were found to contain colistin-resistant *E. coli* isolates. All the animals had received a treatment including colistin for 15 days after the weaning period. Out of the 8 colistin-resistant *E. coli* isolates, only a single isolate (I112) was positive by PCR for the *mcr-3* gene. The other colistin-resistant *E. coli* isolates remained negative for other *mcr*-like genes. Sequencing of the PCR products revealed that the *mcr-3*-like gene corresponded to a new variant, named *mcr-3.12* (GenBank accession number MG564491), encoding the MCR-3.12 enzyme, which shared 97% amino acid sequence identity with the original MCR-3 variant and between 97% and 99% amino acid sequence identity with the other MCR-3-like variants (Fig. 1). Isolate I112 showed resistance to broad-spectrum cephalosporins, tetracycline, chloramphenicol, florfenicol, nalidixic acid, sulfonamides, sulfamethoxazole-trimethoprim, and kanamycin. It was found to be positive by the Rapid Polymyxin NP test and showed a colistin MIC of 4 μg/ml, determined using the broth microdilution method. Multilocus sequence typing (MLST) analysis showed that isolate I112 belonged to sequence type 64 (ST641) and to phylogroup A. Analysis with the SerotypeFinder program (version 1.1) indicated that it belonged to the O160:H25 serotype. Phylogenetic analysis of the known *mcr-3* gene

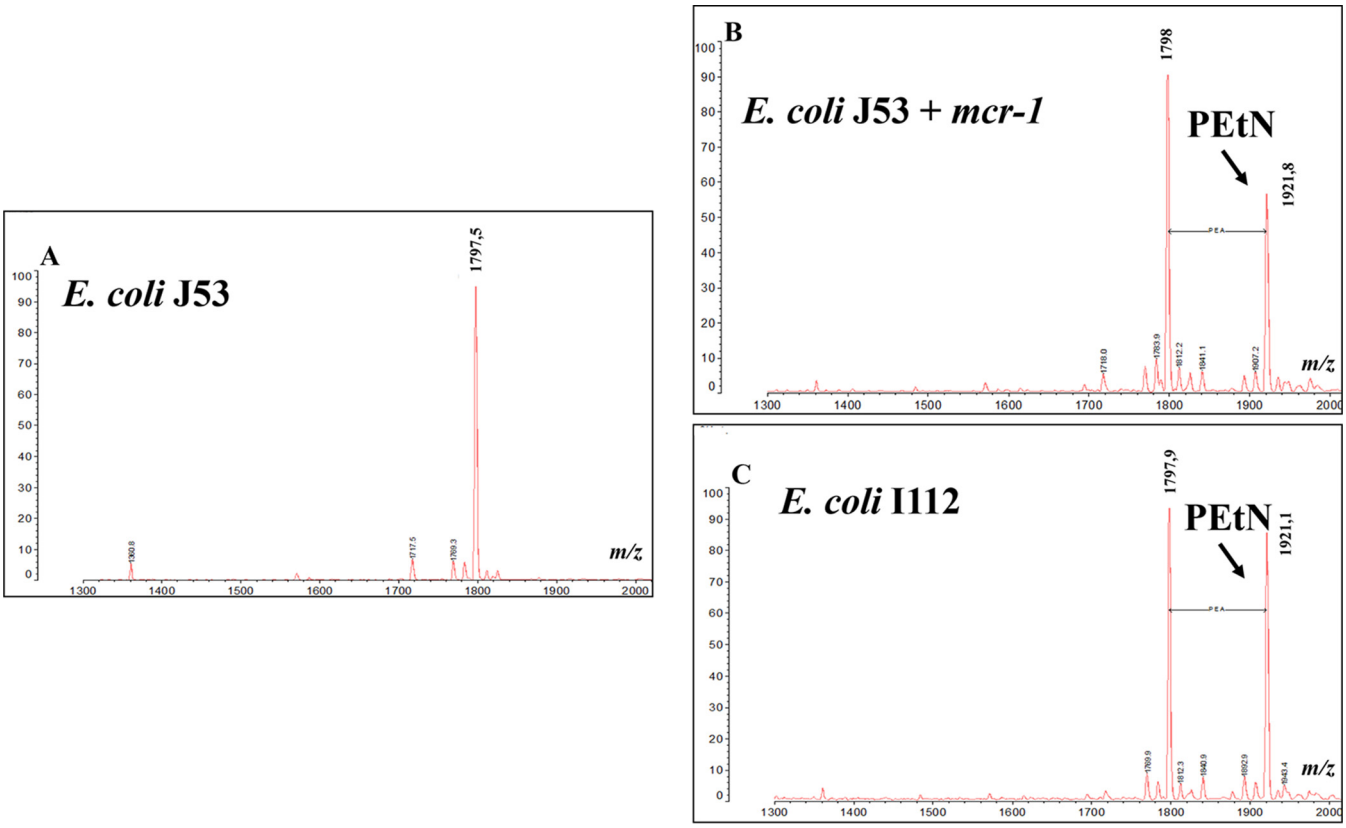

FIG 2 Mass spectrometry analysis of lipid A from strain *E. coli* J53 (A), its transconjugant carrying the *mcr-1* gene (B), and clinical isolate I112 expressing the *mcr-3.12* gene (C). The addition of a PEtN group is indicated by an arrow.

showed a significant diversity among the variants. Three major subgroups could be identified, including (i) MCR-3.5, MCR-3.6, and MCR-3.8, (ii) MCR-3.4 and MCR-3.11, and (iii) MCR-3.1, MCR-3.2, MCR-3.3, MCR-3.7, and MCR-3.11, respectively. Both the MCR-3.9 and MCR-3.10 enzymes were found to be close to the MCR-3.12 and MCR-3.1 variants (Fig. 1).

MCR-3 is a phosphoethanolamine transferase conferring resistance to colistin. Mass spectrometry analysis of the LPS showed that unlike the negative control, strain J53, showing a single *m/z* 1798 peak corresponding to the bis-phosphorylated hexa-acylated lipid A, the MCR-1 and MCR-3 producers showed an identical additional peak at *m/z* 1921 (change in *m/z*, *m/z* 123), corresponding to addition of a phosphoethanolamine (PEtN) group to lipid A, as was previously described (7, 16) (Fig. 2). Induction of the pBAD$_b$-*mcr-3*-like plasmid allowed a colistin MIC of 4 μg/ml to be obtained, whereas the noninduced clone presented a colistin MIC of 0.03 μg/ml, showing that the production of MCR-3.12 conferred a 130-fold increase in the colistin MIC. Altogether, these results show the phosphoethanolamine transferase activity of the MCR-3.12 enzyme and its impact on colistin susceptibility.

Plasmid analysis. Mating-out assays with *E. coli* J53 and *Klebsiella pneumoniae* CIP53153 as the recipients, but also with *Aeromonas punctata* CIP102629 as the recipient, were successful, highlighting the broad host range of the plasmid carrying the *mcr-3.12* variant. In contrast, no transconjugant was obtained using *Pseudomonas aeruginosa* PAO1 as the recipient. Conjugation followed by PCR showed that *mcr-3.12* was located on a conjugative plasmid named p112. That latter plasmid carries genes for resistance to tetracyclines, sulfonamides, chloramphenicol, and florfenicol. PCR-based replicon typing (PBRT) analysis showed that plasmid p112 belongs to the IncA/C$_2$ incompatibility group. Kieser extraction followed by gel electrophoresis identified its size to be ca. 140 kb. The MICs of colistin for the *E. coli* and *K. pneumoniae* transcon-

jugants were 4 and 8 μg/ml, respectively, and therefore, the transconjugants were categorized as resistant according to the EUCAST breakpoint (the original MICs for the bacterial hosts were 0.25 and 0.12 μg/ml, respectively) (http://www.eucast.org). Interestingly, the MIC of colistin for the *A. punctata* transconjugant was 16 μg/ml (original MIC, 0.12 μg/ml), indicating a very significant impact of MCR-3.12 on colistin susceptibility in that species.

Bioinformatic analysis and genetic context of the *mcr-3.12* gene. Whole-genome sequencing of *E. coli* I112 identified a series of resistance determinants, including genes encoding resistance to β-lactams (the bla_{TEM-1B} and $bla_{CTX-M-8}$ genes), aminoglycosides (*aph[3′]-Ia*, *strA*, and *strB*), tetracyclines (*tetA*), phenicols (*catA1* and *floR*), sulfonamides (*sul2*), and trimethoprim (*dfr18*). The *mcr-3*-like gene was found in association with a gene encoding a diacylglycerol kinase *dgkA*-like gene sharing 98% nucleotide sequence identity with the *dgkA* gene identified in association with the first *mcr-3* gene described on plasmid pWJ1 (4).

The *mcr-3.12* gene was located between two insertion sequences belonging to the IS*66* and IS*30* families, respectively (Fig. 3). Interestingly, an inverted repeat left (IRL)-like sequence of IS*66* was detected 90 bp after the end of the inverted repeat right (IRR) sequence of the IS*30*-like sequence and was found to share 100% nucleotide sequence identity with the first 24 nucleotides (nt) of the IRL-like sequence of IS*66* (Fig. 3). This IRL-like sequence downstream of the IS*30*-like sequence could form a putative transposon with IS*66*.

Further analysis showed that this putative transposon was embedded in a longer structure that was inserted between nucleotides 1049 and 1050 of a DNA methyltransferase gene located on the IncA/C_2 backbone. This structure was 20,376 bp long and is represented in Fig. 3F. It could be defined into three different regions: (i) a 5′ region characterized by a 7,666-bp region with a GC content of 39% containing three putative open reading frames (ORFs), including two encoding putative site-specific integrases, (ii) the putative transposon containing the *mcr-3* variant and three ORFs (α, β, and γ) presenting a GC content of 49%, and (iii) a 3′ region of 526 bp with a GC content similar to that of the first 7,666-bp region (Fig. 3F). ORFs α, β, and γ encoded a reverse transcriptase, a transcriptional regulator, and a diguanylate cyclase, respectively. Their products showed strong amino acid sequence identity (98%) with the sequences of putative proteins from *Aeromonas dhakensis*.

DISCUSSION

We report here the identification of a novel variant of the *mcr-3* gene, detected in an *E. coli* isolate recovered from a pig in Brazil. Interestingly, previous studies also described MCR-3 producers recovered from animal samples (11, 13), suggesting the same link between animal and colistin resistance that has been established for the *mcr-1* gene. The pigs screened in this study had previously been treated with colistin for 15 days after the weaning period. This suggests the possible selection of the colistin-resistant strain during this period, as we showed in our previous study describing a high prevalence of MCR-1 producers in a pig farm in Portugal where animals had received colistin (17). There have been many reports of MCR producers in Brazil, with MCR-1 being the only variant systematically identified. These isolates were a single *Salmonella enterica* serotype Typhimurium isolate that was recovered from retail meat (18) and *E. coli* isolates recovered from chicken meat (19), from migratory penguins (20), on public beaches (21), or from patients with bloodstream infections (22, 23). Also, KPC-2-producing *E. coli* isolates (24) and KPC-2-producing *Klebsiella pneumoniae* isolates belonging to ST392 and ST437 (25, 26) were identified. A quite extensive study identified a series of 59 MCR-1-producing *E. coli* isolates recovered from humans, chicken, chicken meat, bovine, turkey, swine, and penguin (27). However, we might speculate that most studies have been designed to detect only the *mcr-1* gene so far, and few have investigated the occurrence of the most recently identified other variants.

Isolate I112 carried a novel *mcr-3* variant named *mcr-3.12*. It belonged to ST641, isolates of which corresponding to isolates recovered from pigs in Germany in 2016

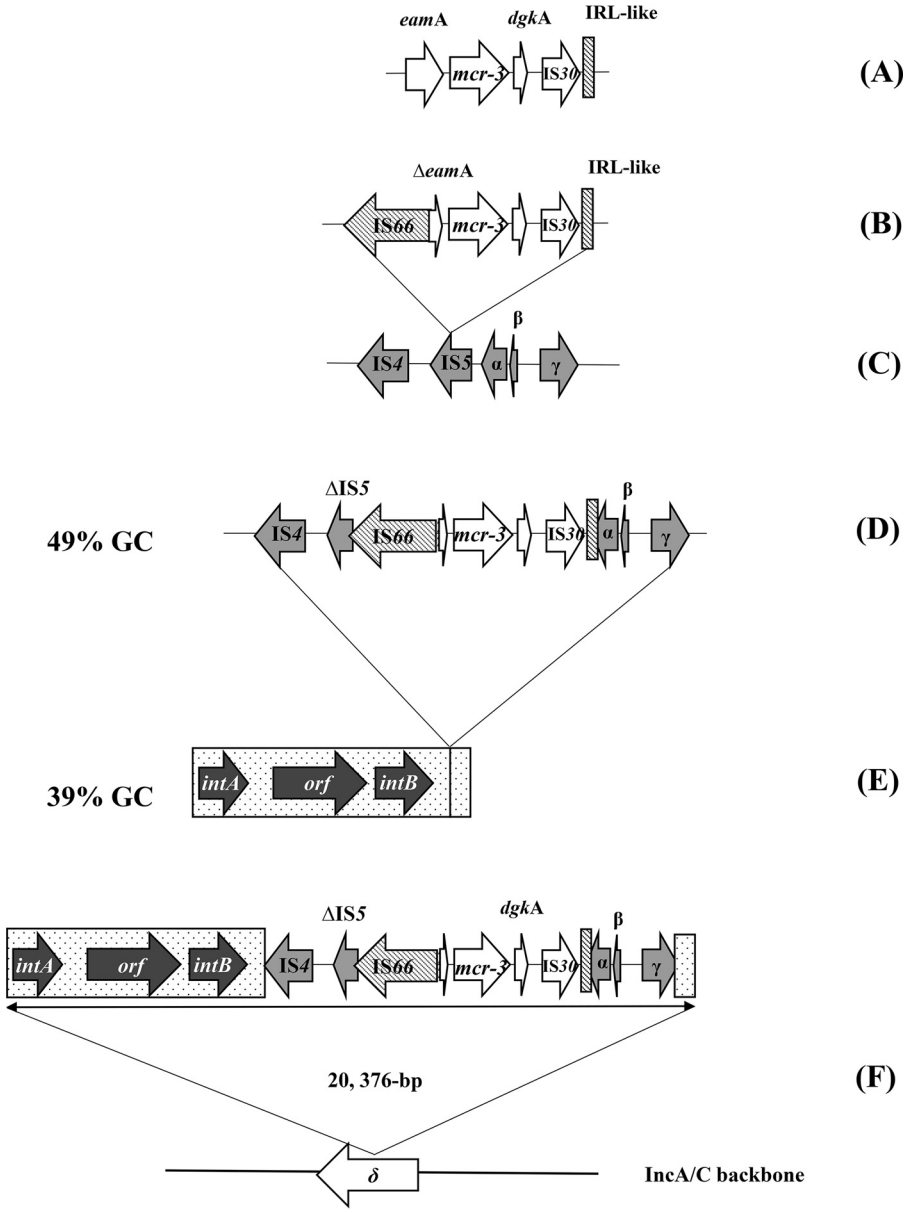

FIG 3 Proposed model of the chronology of the acquisition of the *mcr-3.12* gene into the IncA/C_2 plasmid. The genes *eamA* and *dgkA* encode a metabolite transporter and a diacylglycerol kinase, respectively. *intA* and *intB* represent putative integrases; α, β, and γ are ORFs encoding a reverse transcriptase, a transcriptional regulator, and a diguanylate cyclase, respectively; δ corresponds to the ORF encoding a DNA methyltransferase located on the IncA/C_2 plasmid backbone.

were previously found to carry the *mcr-1* gene (28). It belongs to phylogroup A of *E. coli* and therefore corresponds to a commensal strain. Sequence alignment analysis showed that *mcr-3.12* shares 99% nucleotide sequence identity with the sequence from an *Aeromonas veronii* isolate. This suggests that this new variant may have originated from that particular species or may have widely disseminated as an acquired resistance trait within that species. It is noteworthy that we showed here that the IncA/C_2-type plasmid bearing the *mcr-3.12* gene could replicate in *Aeromonas* spp. We may therefore speculate that such a plasmid type might have been involved in the original spread of *mcr-3*-like genes from their progenitors to other bacterial species, including members of the family *Enterobacteriaceae*.

The results of induction experiments and analysis of the lipid A of the isolate strongly indicate that the MCR-3 enzyme confers colistin resistance in the same way

that the MCR-1 and MCR-2 enzymes do by adding a phosphoethanolamine group to the lipid A moiety, although this enzyme shared only 45 and 47% amino acid sequence identity with the amino acid sequences of MCR-1 and MCR-2, respectively. The fact that MCR-1, -2, and-3 share similar functions was previously hypothesized through *in silico* protein structure analysis (4).

The *mcr-3* gene was previously described on IncHI2 and IncX4 plasmids, which are commonly found in association with the *mcr-1* and *mcr-2* genes. Here, we have described the first IncA/C_2 plasmid carrying a plasmid-mediated colistin resistance determinant. This plasmid backbone is commonly identified to be a support for many different antibiotic resistance genes. Here, the determinants *tetA*, *sul2*, and *floR*, encoding resistance to tetracycline, sulfonamides, and phenicols, respectively, were also detected on the same plasmid. The broad host range of this plasmid was demonstrated by evidencing its ability to replicate not only in *E. coli* and *K. pneumoniae* but also in *A. punctata*.

The *mcr-3.12* gene is located in a putative transposon including the IS*66* upstream sequence of the *mcr-3* gene and an IS*30*-like downstream sequence. Interestingly, a 24-nt region found 90 bp downstream of the IS*30*-like sequence was found to be identical to the IRL of IS*66*. Further experiments will be conducted to confirm whether IS*66* could have played a role in the acquisition of this phosphoethanolamine transferase gene by a mechanism similar to the one-handed transposition that has been described for IS*Ecp1* in the mobilization of $bla_{CTX\text{-}M\text{-}15}$ (29).

The genetic context of the putative *mcr-3* transposon is complex, and the chronology of acquisition of this structure by the IncA/C_2 plasmid can hardly be explained. One hypothesis is summarized in Fig. 3. IS*66* might have been involved in the original mobilization of the *mcr-3.12* gene from *Aeromonas* spp. (Fig. 3A to D). Then, a second mobilization event involving an unknown mechanism between the genetic structure containing the putative integrases (Fig. 3E) and the *mcr-3*-carrying structure may have occurred, forming a 20,376-bp integron-like genetic complex. Finally, this whole structure may have been mobilized and inserted between nt 1049 and nt 1050 of a DNA methyltransferase gene located on an IncA/C_2 plasmid backbone (Fig. 3F). The resulting resistance plasmid is, in the end, one of those responsible for the spread of *mcr* genes among the *Enterobacteriaceae*.

MATERIALS AND METHODS

Bacterial isolate and susceptibility testing. Screening of colistin-resistant isolates from 126 different pigs in 10 swine herds from different sites in the state of Minas Gerais in Brazil was performed. All pigs presented with postweaning diarrhea. The isolates were initially tested for colistin resistance using agar dilution methods. All colonies growing on plates supplemented with >2 μg/ml of colistin were confirmed by the commercialized Rapid Polymyxin NP test (ELITech Microbiology, France) (30), and MICs were determined by the broth microdilution method using cation-adjusted Mueller-Hinton (MH) broth. Antimicrobial susceptibility testing for determination of susceptibility to other antibiotic families was performed according to the standard disk diffusion method on MH agar plates following CLSI recommendations (31).

Whole-genome sequencing and molecular analysis. PCR screening for *mcr* genes was performed using primers designed to detect all known variants of MCR-3. Primers MCR-3allF (5′-GCA TTT ATG CTG AAC TGG CG-3′) and MCR-3allR (5′-AGC GGC TTT CTG CTG CAA AC-3′) were used, and the corresponding amplicons were subsequently sequenced (Microsynth, Balgach, Switzerland). Whole genomic DNA of the MCR-3-positive isolate was extracted by use of a Sigma-Aldrich GenElute bacterial genomic DNA kit. Genomic libraries were assessed using a Nextera XT library preparation kit (Illumina Inc., San Diego, CA), and sequencing was performed using an Illumina MiniSeq system with 300-bp paired-end reads and a coverage of 50 times. The generated FastQ data were compiled and analyzed using the CLC Genomic Workbench (version 7.5.1; CLC Bio, Aarhus, Denmark). Reads were *de novo* assembled with automatic bubble and word size, and contigs with a minimum contig length of 800 nucleotides were generated using the mapping mode map reads back to contigs.

The resulting contigs were uploaded into the Center for Genomic Epidemiology server (http://www.genomicepidemiology.org/). The plasmid replicon type, multilocus sequence type, serotype, and antimicrobial resistance determinants were determined using the PlasmidFinder (version 1.3), MLST (version 1.8), SerotypeFinder (version 1.1), and ResFinder (version 3.0) programs, respectively (32–34). Phylogroup analysis was performed by using the method described by Clermont et al. (35). Sequence alignments and construction of phylogenetic trees were performed with the SeaView alignment tool (version 4; Prabi, La Doua, Lyon, France) (36).

Plasmid analysis was performed using the Kieser extraction method (37) followed by gel electrophoresis in order to estimate the size of the plasmid containing the *mcr-3* gene using *E. coli* strain 50192 harboring four plasmids of 154, 66, 48, and 7 kb, respectively, as the plasmid size marker. Determination of the incompatibility group was confirmed by PCR-based replicon typing (PBRT) (38).

Conjugation experiments were performed using the azide-resistant strain *E. coli* J53. In addition, conjugations were also performed with temocillin-resistant *Pseudomonas aeruginosa* strain PAO1, azide-resistant *Klebsiella pneumoniae* strain CIP53153, and azide-resistant *Aeromonas punctata* strain CIP102629 as the recipient strains to test the plasmid carrying the *mcr-3.12* variant for a broad host range. Both donor and recipient strains were cultured in exponential phase and then mixed on solid LB agar using filters at a 1:10 donor/recipient ratio. After 5 h of incubation, the filters were resuspended in 0.85% NaCl and the bacterial mixture was plated onto agar plates supplemented with colistin (1 μg/ml) and sodium azide (100 μg/ml) for *E. coli* or with temocillin (50 μg/ml) and sodium azide (100 μg/ml) for *P. aeruginosa*. Since the plasmid bearing the *mcr-3.12* gene conferred resistance to tetracycline, conjugations using *K. pneumoniae* and *A. punctata* as the recipients were attempted using tetracycline (100 μg/ml) and sodium azide (100 μg/ml) as selective molecules. The susceptibility of all transconjugants to antibiotics was confirmed by use of the antibiogram followed by PCR for the *mcr-3*-like gene.

Analysis of the LPS modification. The LPS of *E. coli* J53 (unmodified lipid A), TCAf24 (J53 *mcr-1* transconjugant), and I112 (MCR-3-like producer) were analyzed by mass spectrometry (MS). Lipid A was obtained by hydrolysis of 3 mg of lyophilized bacteria in 120 μl of isobutyric acid and 1 M ammonium hydroxide (5:3; vol/vol), heating for 1 h at 100°C, and cooling at 4°C before centrifugation, as previously described (39). The supernatant was then diluted with water and lyophilized before it was washed with methanol. The insoluble lipid A obtained was finally extracted in a chloroform-methanol-water (3:1:0.25, vol/vol/vol) mixture. Matrix-assisted laser desorption ionization-MS analysis was performed using a PerSeptive Voyager STR (PE Biosystems, France) time of flight mass spectrometer in linear negative-ion mode. Dihydroxybenzoic acid (DHB) at 10 mg/ml in 0.1 M citric acid in chloroform-methanol-water (3:1.5:0.25, vol/vol/vol) was used as the matrix.

Cloning and overexpression of the *mcr-3.12* gene. The new *mcr-3* variant was cloned into the arabinose-inducible $pBAD_b$ vector in order to determine the impact of the expression of the MCR-3.12 phosphoethanolamine transferase on colistin susceptibility. Induction of the $pBAD_b$ vector was performed using MH broth supplemented with 1% L-arabinose as previously described (8).

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